Evaluation of mucositis induced by irinotecan after microbial colonization in germ-free mice

Silvia H. S. P. Pedroso,1† Angélica T. Vieira,1,2† Rafael W. Bastos,1 Jamil S. Oliveira,2 Christiane T. Cartelle,3 Rosa M. E. Arantes,3 Pedro M. G. Soares,4 Simone V. Generoso,5 Valbert N. Cardoso,6 Mauro M. Teixeira,2 Jacques R. Nicoli1 and Flaviano S. Martins1

1Department of Microbiology, Biological Science Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
2Department of Biochemistry and Immunology, Biological Science Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
3Department of General Pathology, Biological Science Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
4Department of Morphology, Medical School, Federal University of Ceará, Fortaleza, Ceará, Brazil
5Department of Basic Nursing, School of Nursing, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
6Department of Clinical and Toxicological Analysis, Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Mucositis is one of the most debilitating side effects of chemotherapy and some previous studies suggest a role for indigenous microbiota in the course of this pathology. Therefore, the aim of our study was to evaluate the differences in phenotype between germ-free (GF) and conventional (CV) mice, and the role of β-glucuronidase-producing bacteria in the development of irinotecan treatment in a murine model. After mucositis induction, CV mice showed a significant increase in all inflammatory parameters when compared to GF mice. CV animals also showed more lesions of the intestinal epithelium, coherent with their higher intestinal permeability. The conventionalization of GF animals reversed their phenotype to that found in CV mice. In addition, gnotobiotic mice monoassociated with an Escherichia coli strain producing β-glucuronidase showed an increased permeability when compared to gnotobiotic mice monoassociated with an E. coli strain deleted for the gene encoding β-glucuronidase, but these did not show any differences in the influx of neutrophils, eosinophils or histological characteristics. Our data confirmed that components of the gut microbiota are involved in the signs of mucositis. Nevertheless, other mechanisms than this enzyme are involved in the irinotecan treatment, since the monoassociation was not able to restore the entire phenotype observed in the CV animals with irinotecan treatment in our murine model.

INTRODUCTION

Mucositis refers to a mucosal damage of the gastrointestinal tract (GIT) secondary to cancer therapy. This pathology has a great prevalence in almost all patients undergoing high doses of chemotherapy and the main symptoms include abdominal pain, diarrhoea, constipation and weight loss (Lalla et al., 2014; van Vliet et al., 2010). These symptoms are frequently serious enough to justify interruptions or reductions of cancer treatment, leading to an increase in...
the mortality rate of patients. The pathophysiology of mucositis is still not fully understood and there are no effective treatments (Sonis, 2004). The GIT is particularly susceptible to cancer therapy due to its high rate of cell renewal, which is characterized by inflammation and loss of epithelial barrier integrity (Dekaney et al., 2009).

Camptothecin (CPT)-11 (irinotecan) is an antiproliferative drug and has been widely used for cancer treatment, particularly for colorectal cancer, exerting its cytotoxic effect by inhibiting DNA topoisomerase I (Lee et al., 2014; Vanhoefer et al., 2001). To act, CPT-11 requires conversion to its active metabolite 7-ethyl-10-hydroxy camptothecin (SN-38) by a hepatic carboxylesterase (Voigt et al., 1998). This metabolite has been described as inducing collateral intestinal damage (Alimonti et al., 2003), and its detoxification occurs primarily in the liver by UDP-glucuronosyltransferase 1A1 (UGT1A1)-catalysed glucuronidation to form the inactive SN-38 glucuronide (SN-38G), which subsequently undergoes biliary excretion.

The intestinal microbiota seems to play a dual role in the development of mucositis. On one hand, beneficial bacteria produce metabolites, such as butyrate, that are beneficial to the colonic mucosa and reduce mucositis (Ramos et al., 1997); this is in contrast to β-glucuronidase-producing bacteria (such as enterobacteria) that deconjugate SN-38G back to the toxic metabolite SN-38 in the intestine, which is considered to be responsible for CPT-11-associated intestinal damage. This hypothesis is reinforced by the increased concentrations of CPT-11 observed in the GIT of animals with chemotherapy-induced mucositis (Stringer et al., 2007, 2009a). Together, these findings strongly suggested that the intestinal microbiota may be involved in the pathophysiology of irinotecan treatment. However, studies evaluating the direct influence of intestinal microbiota on the pathogenesis of mucositis are very scarce. Thus, in this study, we evaluated the influence of indigenous microbiota on intestinal pathology associated with mucositis by comparing histopathological, immunological and physiological parameters in germ-free (GF), conventional (CV), conventionalized (CVO) and gnotobiotic mice monoassociated with bacteria producing or not producing β-glucuronidase and receiving intraperitoneal (i.p.) irinotecan.

**METHODS**

**Animals.** GF and CV 6–8-week-old female NIH Swiss mice (Taconic) were used in this work. Water and commercial autoclavable diet (Nuvital) were sterilized by steam and administered ad libitum. GF mice were housed in flexible plastic isolators (Standard Safety Equipment Company) and handled according to established procedures. Experiments with gnotobiotic monoassociated mice were carried out in micro-isolators (Uno Roestvastaal). Monoassociated and CV mice were housed in an animal facility with controlled lighting (12 h light–dark cycle), humidity (60–80 %) and temperature (22 ± 1 °C). All experimental procedures were carried out according to the standards set forth by the Brazilian Society of Laboratory Animal Science/Brazilian College for Animal Experimentation (available at: http://www.cobea.org.br/) and the study was approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CEUA/UFRG protocol no. 186/2012). A total number of ten animals per group were used in this study (four for histological analysis and six for the other parameters) in each experiment. As a microbiological control, experiments with GF or gnotobiotic monoassociated mice were tested by inoculating with recently collected faeces and incubating at 37 °C in thioglycollate broth and brain heart infusion media (BHI) broth media, and by Gram staining of the faeces.

**Mucositis induction.** Mice undergoing experimental mucositis were i.p. injected with 75 mg CPT-11 kg⁻¹ (irinotecan hydrochloride, Evoteron; Evolabis) on days 0, 1 and 2, and sacrificed on day 5 after the first dose. Control mice received saline by i.p. injection. This protocol was adapted from that of Ikuno et al. (1995).

**Experimental design.** Mice were randomized into 10 groups: (i) CV control (CV) – received i.p. saline; (ii) GF control (GF) – received i.p. saline; (iii) CVO control (CVO) – received orally fresh diluted faeces from CV and i.p. saline; (iv) CV (CV+CPT-11) – received i.p. irinotecan; (v) GF (GF+CPT-11) – received i.p. irinotecan; (vi) CVO (CVO+CPT-11) – received orally fresh diluted faeces from CV and i.p. irinotecan; (vii) gnotobiotic mice orally monoassociated with Escherichia coli producing β-glucuronidase (MN-TG1) – received i.p. saline; (viii) gnotobiotic mice orally monoassociated with E. coli producing β-glucuronidase (MN-TG1+CPT-11) – received i.p. irinotecan; (ix) gnotobiotic mice orally monoassociated with E. coli strain deleted for the gene encoding β-glucuronidase (MN-191) – received i.p. saline; (x) gnotobiotic mice orally monoassociated with E. coli strain deleted for the gene encoding β-glucuronidase (MN-191+CPT-11) – received i.p. irinotecan. Before sacrifice, mice were anaesthetized (60 : 80 mg kg⁻¹, ketamine : xylazine, i.p.; Syntec) for blood collection, and then sacrificed by cervical dislocation to collect the small intestine, blood and intestinal fluid for further analyses.

**Conventionalization.** The repositioning of microbiota was achieved by oral administration of fresh diluted faeces from CV mice, as briefly described below (Souza et al., 2004). Faeces removed from the intestine of CV mice were homogenized and diluted in saline (10⁻¹) and 100 µl of this suspension was administered to GF mice through oral gavage. After 21 days, CVO mice were submitted to irinotecan treatment, as described above. Thioglycollate tests showed a similar number of bacteria in CVO and CV mice (data not shown).

**Histological analysis of the jejunum.** Samples (small intestine) were fixed in 4 % formaldehyde and processed by inclusion in paraffin for microtomy. They were washed in running water for 5 min, then the sections were dipped in an aqueous solution of 0.5 % periodic acid for 5 min, followed by incubation in Schiff reagent for 10 min, then they were counterstained with Harris haematoxylin and lastly they were washed in running water for light staining (12 h light–dark cycle), humidity (60–80 %) and temperature (22 ± 1 °C). All experimental procedures were carried out according to the standards set forth by the Brazilian Society of Laboratory Animal Science/Brazilian College for Animal Experimentation (available at: http://www.cobea.org.br/) and the study was approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CEUA/UFRG protocol no. 186/2012). A total number of ten animals per group were used in this study (four for histological analysis and six for the other parameters) in each experiment. As a microbiological control, experiments with GF or gnotobiotic monoassociated mice were tested by inoculating with recently collected faeces and incubating at 37 °C in thioglycollate broth and brain heart infusion media (BHI) broth media, and by Gram staining of the faeces.

**Mucositis induction.** Mice undergoing experimental mucositis were i.p. injected with 75 mg CPT-11 kg⁻¹ (irinotecan hydrochloride, Evoteron; Evolabis) on days 0, 1 and 2, and sacrificed on day 5 after the first dose. Control mice received saline by i.p. injection. This protocol was adapted from that of Ikuno et al. (1995).

**Experimental design.** Mice were randomized into 10 groups: (i) CV control (CV) – received i.p. saline; (ii) GF control (GF) – received i.p. saline; (iii) CVO control (CVO) – received orally fresh diluted faeces from CV and i.p. saline; (iv) CV (CV+CPT-11) – received i.p. irinotecan; (v) GF (GF+CPT-11) – received i.p. irinotecan; (vi) CVO (CVO+CPT-11) – received orally fresh diluted faeces from CV and i.p. irinotecan; (vii) gnotobiotic mice orally monoassociated with Escherichia coli producing β-glucuronidase (MN-TG1) – received i.p. saline; (viii) gnotobiotic mice orally monoassociated with E. coli producing β-glucuronidase (MN-TG1+CPT-11) – received i.p. irinotecan; (ix) gnotobiotic mice orally monoassociated with E. coli strain deleted for the gene encoding β-glucuronidase (MN-191) – received i.p. saline; (x) gnotobiotic mice orally monoassociated with E. coli strain deleted for the gene encoding β-glucuronidase (MN-191+CPT-11) – received i.p. irinotecan. Before sacrifice, mice were anaesthetized (60 : 80 mg kg⁻¹, ketamine : xylazine, i.p.; Syntec) for blood collection, and then sacrificed by cervical dislocation to collect the small intestine, blood and intestinal fluid for further analyses.

**Conventionalization.** The repositioning of microbiota was achieved by oral administration of fresh diluted faeces from CV mice, as briefly described below (Souza et al., 2004). Faeces removed from the intestine of CV mice were homogenized and diluted in saline (10⁻¹) and 100 µl of this suspension was administered to GF mice through oral gavage. After 21 days, CVO mice were submitted to irinotecan treatment, as described above. Thioglycollate tests showed a similar number of bacteria in CVO and CV mice (data not shown).

**Histological analysis of the jejunum.** Samples (small intestine) were fixed in 4 % formaldehyde and processed by inclusion in paraffin for microtomy. For analysis of intestinal lesions, the tissues were dipped in periodic acid for 5 min, followed by incubation in Alcian blue (pH 2.5), and then washed in running water for 5 min, then the sections were dipped in the equilibrium solution (3 % acetic acid) and fixed in Bouin solution as described by Arantes & Nogueira (1997). Sections 4–5 µm thick were stained with haematoxylin/eosin (HE), coded and analysed through optical microscopy. The images were obtained by a JVC TK-1270/RGB microcamera and the KS 300 software built into a Kontron Elektronick/Carl Zeiss image analyser.

**Periodic acid Schiff/Alcian blue staining.** After being deparaffinized, jejunum sections were processed according to Cawley et al. (1956). They were washed in running water for 5 min, then the sections were dipped in the equilibrium solution (3 % acetic acid) for 3 min; afterwards they were immersed in 1 % Alcian blue (pH 2.5), and finally washed with equilibrium solution and distilled water. After these procedures, the samples were dipped in an aqueous solution of 0.5 % periodic acid for 5 min, followed by incubation in Schiff reagent for 10 min, then they were counterstained with Harris haematoxylin and lastly they were washed in running water for
15 min. After staining, sections were dehydrated and mounted on slides with Entellan (Merck).

**Immunohistochemistry for proliferating cell nuclear antigen (PCNA).** After being deparaffinized, slides containing jejunum sections were rehydrated and placed in 10 % ammonium hydroxide with 95 % ethanol to remove formalin pigment, then washed with distilled water, followed by antigen retrieval with citrate buffer for 20 min. The next steps were the blocking of endogenous peroxidase using methanol/hydrogen peroxide for 30 min, with 2 % BSA for 30 min, and with biotin/avidin for 10 min, all steps being performed at room temperature. Then, the primary antibody PCNA (rabbit mAb; Epitomics Abcam), 1 : 200, was pipetted onto the sections and incubated overnight. The secondary antibody (anti-rabbit, anti-mouse and anti-goat immunoglobulin) streptavidin peroxidase biotinylated (LSAB2 system-HRP kit; Dako) was used consecutively for 30 min at room temperature. The development reaction was performed by incubation in DAB solution (50 mg diaminobenzidine in 200 ml PBS and 400 µl 30 % hydrogen peroxide) for 5 min. Subsequently, these sections were counterstained with Harris haematoxylin for 3 s, then washed in running water for 5 min, dehydrated in increasing concentrations of alcohol, diaphanized in xylene and mounted on Entellan slides (Merck).

**Intestinal permeability.** The intestinal permeability was evaluated according to Maioli et al. (2014). All mice received 0.1 ml diethyl-enediaminetetraacetic acid (Nuclear) solution labelled with 18.5 MBq of 99m-technetium by gavage. After 4 h, the animals were anaesthetized and 500 µl blood was collected and placed in appropriate tubes for radioactivity determination using a scintillator. Data were expressed as c.p.m. (g blood)⁻¹.

**Determination of slgA levels.** The small intestine was removed by cutting the gastroduodenal and ileocaecal junction. The contents were removed, weighed and diluted with PBS, pH 7.2, containing anti-proteases in a ratio of 500 mg content : 2.0 ml PBS. Then, they were centrifuged (2000 g, 30 min, 4 °C) and the supernatant collected and frozen at −80 °C for later determination of immunoglobulins. The determination of total slgA levels was performed by ELISA as previously described by Martins et al. (2009).

**Determination of the eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activity in the jejenum.** The extent of tissue eosinophil infiltration was assessed by measurement of EPO activity, as described below (Vicira et al., 2009). Every 100 mg jejenum was homogenized with 1.9 ml PBS and centrifuged at 12 000 g for 10 min. The supernatant was discarded, and the erythrocytes were lysed. The samples were then centrifuged, the supernatant discarded and the pellet resuspended in 1.9 ml of 0.5 % hexadecyltrimethylammonium bromide in PBS; this was followed by three cycles of freezing in liquid nitrogen, and finally centrifugation at 12 000 g under 4 °C for 10 min. The supernatant was used in the enzymic assay with the addition of an equal amount of substrate (1.5 mM o-phenylenediamine and 6.6 mM H₂O₂ in 0.075 mM Tris/HCl pH 8). The reaction was stopped with 50 µl 1 M H₂SO₄, and the absorbance was read at 492 nm.

The extent of neutrophil accumulation in the jejenum tissue was measured by assaying MPO activity, as described below (Elian et al., 2015). Portions of the jejenum were removed and snap frozen in liquid nitrogen. After thawing and processing, the tissues were assayed for MPO activity by measuring the change in absorbance at 450 nm using tetramethylbenzidine.

**Determination of cytokine levels in the jejenum.** Cytokine levels were measured according to Elian et al. (2015). This is briefly described as follows. The small intestine was homogenized with PBS (0.4 M NaCl, 10 mM Na₂HPO₄), containing anti-proteases (0.1 mM PMSF, 0.1 mM benzthonium chloride, 10 mM EDTA and 20 Kallikrein Inhibitor Units aprotinin A) and 0.05 % Tween 20. The samples were then centrifuged for 10 min at 12 000 g and the supernatant was used immediately for assays. The concentration of cytokines (IL-1β, TNF-α and IL-10) was measured by ELISA using commercially available antibodies and according to the procedures supplied by the manufacturer (R&D Systems).

**Determination of SN-38 in intestinal fluid by HPLC.** To determine the amount of SN-38 in the intestinal fluid, the methodology proposed by Bansal et al. (2008) was followed with some modifications. Primary stock solutions for SN-38 (Sigma Aldrich) were prepared separately in DMSO at a concentration of 1 mg ml⁻¹. Working standard solutions were prepared daily by serial dilution in acetonitrile containing 0.1 % glacial acetic acid, to obtain serial concentrations from 100 to 0.001 µg ml⁻¹. Feasibilities of mixtures of various solvents such as ethyl acetate, dichloromethane (DCM), tert-butylmethylether (TBME), diethyl ether and n-hexane were evaluated for their use in extraction of samples from intestinal fluid. A 1 ml aliquot of DCM : TBME (3 : 7) was added, and the mixture was vortexed and then shaken for 10 min. Subsequently, centrifugation (3000 g for 10 min) was performed to separate the aqueous and organic layer. A 900 µl aliquot of the organic layer was transferred to a microtube and evaporated until dry under vacuum at 40 °C. The dried extract was redissolved by vortexing and sonication in 150 µl 10 : 90 (v/v) acetonitrile : water (pH 3.0). Lastly, a 150 µl volume was injected into the HPLC system (LC-20AD/T LPGE kit, Shimadzu).

**Monoassociation of GF mice with E. coli producing β-glucuronidase or with an E. coli strain deleted for the gene encoding β-glucuronidase.** The bacterial strains used in this study were E. coli TG1 (supE thiD lac-proAB hsdS F⁻ traD36 proAB lacIZ515) and E. coli L91 (the TG1 strain with ΔuidA : Km⁻¹) (Beaud et al., 2005). The TG1 strain, which has an active β-glucuronidase, was used to produce an isogenic derivative strain inactivated for this enzymic activity (named L91). Both strains were kindly provided by Dr Jamila Anba-Mondoloni, Unité Mixte de Recherche MICALIS, Institute National de la Recherche Agronomique (INRA), Jouy-en-Josas, France. A growth curve of the two bacterial strains showed that both have the same growth rate. Therefore, a 0.5 ml suspension containing 10⁷ cells was administered orally to GF animals and, after 5 days, mucositis was induced according to previously described methodology. Both bacteria reached the same number/colonization levels (as observed by diluting and plating faeces onto BHI agar). Twenty-four hours before mucositis induction, both bacteria reached 10⁵ c.f.u. (g faeces)⁻¹.

**Statistical analysis.** The results were expressed as the mean of at least two independent experiments. Results are shown as the mean ± SEM. Normalized data were compared by using ANOVA, followed by a Tukey post hoc analysis. Results were considered significant at P < 0.05. The tests were performed using the statistical software Prism.

**RESULTS**

**GF animals were less susceptible to irinotecan treatment**

The induction of mucositis resulted in important changes in intestinal histology in CV animals, such as: loss of the mucosa’s structure and consequent reduction of its thickness; intense infiltration of polymorphonuclear and mononuclear cells; and loss of cell differentiation (Fig. 1a, b). However, we observed that in the absence of intestinal
microbiota, the changes due to mucositis in GF mice were not observed when compared to the CV group (Fig. 1c, d). In order to confirm these qualitative observations, morphometric analyses were performed. As expected, inflammatory infiltrate (Fig. 1e) and height of the intestinal villi (Fig. 1f) were, respectively, increased and reduced ($P<0.01$) in CV + CPT-11 when compared to the GF group, evidencing higher intestinal damage in CV mice with irinotecan treatment. There was no change in these parameters when GF mice were compared to GF + CPT-11 animals.

In order to analyse the influence of intestinal microbiota and induction of mucositis on the number of goblet cells and the cell proliferation rate of the intestinal epithelium, periodic acid Schiff /Alcian blue staining and immunohistochemistry for PCNA were performed. An increase in the proliferation rate of the intestinal epithelium (Fig. 1g) and a decrease in goblet cells per area of intestinal mucosa (Fig. 1h) were observed when CV mice were compared to CV + CPT-11 mice. In contrast, CV animals showed an elevated proliferation rate of the intestinal epithelium (Fig. 1g, $P<0.01$) and fewer goblet

**Fig. 1.** Effects of microbiota on intestinal mucosa destruction and permeability after irinotecan treatment. Histological aspects of jejunum tissue of mice treated (or not) with irinotecan: (a) CV control, (b) CV treated with irinotecan, (c) GF control, (d) GF treated with irinotecan. Morphometric aspects of jejunum tissue: (e) inflammatory infiltrate cells counts, (f) height of intestinal villi, (g) cell proliferation rate (PCNA), (h) number of goblet cells and (i) intestinal permeability. HE staining (a–d), original magnification $×4$. *$P<0.05$; **$P<0.01$; ***$P<0.001$. 

http://mic.microbiologyresearch.org 1953
cells per area of intestinal mucosa (Fig. 1h, \( P < 0.001 \)) when compared to GF mice with or without irinotecan treatment. These histopathological results were accompanied by an increased intestinal permeability observed in CV animals treated with irinotecan (Fig. 1i, \( P < 0.01 \)). GF mice did not show this alteration in intestinal permeability after irinotecan treatment when compared to GF control mice (Fig. 1i).

To evaluate the cell types present in the inflammatory intestinal infiltrate, we used MPO- and EPO-based activity assays. Since MPO is the most abundant enzyme in neutrophils and EPO is expressed in eosinophils, the values of the activities of these enzymes are considered useful and reliable markers for neutrophil and eosinophil infiltration, respectively (Vieira et al., 2009). An accumulation of neutrophils and eosinophils in the small intestine mucosa of CV mice after irinotecan treatment was observed \( (P < 0.01) \), whereas GF mice did not show such alterations after the induction of mucositis (Fig. 2a, b). We then evaluated the production of some inflammatory cytokines in the small intestine; an increased production of the pro-inflammatory IL-1\( \beta \) and TNF-\( \alpha \) cytokines was observed in CV mice after irinotecan treatment, whereas this phenomenon was not observed in GF animals (Fig. 2c, d). As described in another study, basal levels of the anti-inflammatory cytokine IL-10 were higher in GF than in CV mice (Fagundes et al., 2012). Nevertheless, it fell to CV levels after irinotecan administration (Fig. 2e).

We also measured the levels of IgA, both in intestinal fluid and serum of all CV and GF groups (Fig. S1, available in the online Supplementary Material). While in GF groups (in both control and mucositis groups, in serum and intestinal fluid) IgA was detected in very low concentrations, we observed that in CV animals after irinotecan treatment, levels of sIgA decreased in the intestinal fluid (Fig. S1A) as opposed to the increased levels in serum (Fig. S1B). Altered permeability might be responsible for this inversion, allowing movement of IgA from one compartment to another.

We next quantified the levels of SN-38 – the active metabolite of CPT-11 – in intestinal fluid of GF and CV mice after irinotecan treatment. We observed that GF mice treated with irinotecan showed an increased concentration of SN-38 in intestinal fluid compared to CV mice treated with irinotecan (Fig. 2f). Altogether, these findings indicate that absence of microbiota in GF mice is associated with reduction of intestinal inflammation, pathology and gut permeability after irinotecan treatment.

![Fig. 2](https://www.microbiologyresearch.org)
Conventionalization of GF mice restored the CV phenotype of intestinal pathology and mucosal inflammation after irinotecan treatment

In order to confirm that the intestinal histopathological differences observed between GF and CV mice were due to the intestinal microbiota, the conventionalization of GF mice was carried out with the fresh faeces of CV mice for 21 days. As we hypothesized, the phenotype of CV mice was restored in CVO mice, as demonstrated by histology (Fig. 3a, b). We observed a decrease in jejunum villus height (Fig. 3c) and numbers of goblet cells per area of intestinal mucosa (Fig. 3d) in CVO mice after irinotecan treatment, which was similar to CV mice with irinotecan treatment. Analysing the intestinal cell proliferation by PCNA, we observed increased PCNA in the intestinal mucosa of CVO mice after irinotecan treatment when compared to CVO control mice (Fig. 3e) and this result was similar to the result found in CV mice. It was observed that the inflammatory infiltration was restored in the intestinal mucosa of GF mice after conventionalization and irinotecan treatment (Fig. 3f). As in CV mice, infiltration of cells into the intestine of CVO mice after irinotecan treatment was characterized by accumulation of neutrophils (Fig. 3g) and eosinophils (Fig. 3h).

**Fig. 3.** Effects of conventionalization of GF mice on the phenotype of mucosal inflammation after irinotecan treatment. Histological aspects of jejunum tissue of mice treated (or not) with irinotecan: (a) CVO control, (b) CVO treated with irinotecan. Morphometric aspects of jejunum tissue: (c) height of intestinal villi, (d) number of goblet cells, (e) cell proliferation rate (PCNA), (f) inflammatory infiltrate cells counts, (g) MPO and (h) EPO. HE staining (a, b), original magnification ×4. *P<0.05; **P<0.01; ***P<0.001.
As expected, the intestinal permeability increased in CVO mice in a similar way to CV animals when compared to the GF group after irinotecan treatment (Fig. 4a). Along with the higher intestinal permeability in CVO mice with irinotecan treatment, we detected decreasing levels of SN-38 in the intestinal fluid of CVO irinotecan-treated compared to GF irinotecan-treated mice (Fig. 4b).

In order to confirm that the phenotypes observed in CVO mice were due to the conventionalization, we activated the immune system of GF mice by injecting LPS systemically and then inducing mucositis (Fig. S2). Systemic injection of LPS was not able to restore the CV phenotype in these animals as shown by intestinal pathology and inflammatory cells infiltration (Fig. S2). All these results suggest that the intestinal damages observed in CV mice after mucositis induction were due to the presence of intestinal microbiota.

**Monoassociated β-glucuronidase-producing bacteria increased intestinal permeability in gnotobiotic mice with induced mucositis**

To confirm the role of β-glucuronidase bacteria in induction of mucositis, we analysed the histological parameters, influx of neutrophils, eosinophils and intestinal permeability of GF mice monoassociated with two different E. coli: the wild-type TG1 strain (β-glucuronidase producer) and its L91 mutant, which doesn’t produce the enzyme. Faecal cultures were assessed in order to evince
that both strains had the same ability to colonize the intestinal epithelium. The GF mice monoassociated with the TG1 strain showed an increased intestinal permeability when compared to the group monoassociated with the L91 mutant strain (Fig. 4c), but there were no differences in the other parameters analysed – histopathology, activity of MPO or EPO (data not shown). These results suggest that the enzyme β-glucuronidase also plays a key role in increasing intestinal permeability.

Together, these findings indicate that the intestinal damage observed in the CV animals after irinotecan administration was due to the presence of the gut microbiota. Nevertheless, mechanisms other than the enzymic activity are involved in the irinotecan treatment, since the monoassociation was not able to restore the entire phenotype observed in the CV animals with irinotecan treatment.

DISCUSSION

The major findings of our study comparing GF, CV, CVO and monoassociated mice can be summarized as: (i) in the absence of an intestinal microbiota, we observed a resistance in development of intestinal damage due to irinotecan administration; (ii) conventionalization of GF mice reversed the phenotype from resistant to sensitive to irinotecan treatment; and (iii) the monoassociation of GF mice with β-glucuronidase-producing bacteria increased intestinal permeability after irinotecan treatment. The role of human intestinal microbiota in health and disease is being increasingly appreciated. Although there is a growing amount of literature evaluating the influence of intestinal microbiota in chemotherapy-induced mucositis (Brandi et al., 2006; Stringer et al., 2009b; van Vliet et al., 2009), the relationship between intestinal bacteria and mucositis is poorly described. Interestingly, Brandi et al. (2006) previously showed that GF mice were more resistant to a lethal dose of irinotecan than their CV counterparts.

The GF animal model represents a fundamental tool to explore and elucidate the interaction between the host and its microbiota. In the absence of microbiota, GF animals are generally more susceptible to microbial infections (Fagundes et al., 2012); however, GF status can ensure a higher resistance to some other pathological conditions (generally involving inflammation), in which the indigenous microbiota acts as a trigger to induce the disease (Marteaup & Chaput, 2011; Xavier & Podolsky, 2007). Fagundes et al. (2012) demonstrated that GF mice did not survive a bacterial lung infection due to their inability to effectively process an appropriate inflammatory response. These authors also found a high innate production of IL-10 in GF mice, which may explain, in part, the predominance of the anti-inflammatory response in these animals, as demonstrated by other studies (Souza et al., 2004, 2007). We also showed here that GF mice produced high levels of IL-10 that may explain in a similar way the reduction of inflammatory infiltration and the consequent prevention of intestinal damage after irinotecan treatment in GF mice.

In our work, the intestinal damage induced by irinotecan was characterized by epithelial destruction, decreased size of the villi, increased intestinal permeability and inflammatory infiltration, and high disorganization of epithelial architecture in the intestinal mucosa, as seen in a similar study (Ikuno et al., 1995). These characteristics were observed in the intestine of CV mice with irinotecan treatment, but not in their GF counterparts. Additionally, conventionalization of GF mice before irinotecan treatment was able to increase the intestinal damage in a similar way to CV mice, hence reinforcing the hypothesis that the intestinal microbiota directly participates in the histopathological changes induced by irinotecan.

Neutrophils and eosinophils are among the earliest immune cells recruited to the site of an intestinal injury. The major function of these cells is to protect the host from infection by combating invading micro-organisms and clearing cellular debris. However, activated neutrophils secrete a battery of bioactive substances, such as proteases and reactive oxygen intermediates, which in excess can lead to intestinal tissue damage (Fournier & Parkos, 2012; Vieira et al., 2009). Previous studies have demonstrated the participation of these inflammatory cells in the development of mucositis (Fijlstra et al., 2011; Leitão et al., 2007; Lima et al., 2005). Furthermore, a study has shown that in GF mice subjected to conventionalization, the microbiota installation influences intestinal neutrophil recruitment by Myd88-dependent innate immunity signalling (Bates et al., 2007). The restoration of neutrophil and eosinophil influx into the inflamed intestinal tissue of GF mice that had undergone conventionalization before irinotecan treatment was also observed in our study, showing again that the microbiota seems to be associated with the intestinal damage. However, although conventionalization of GF mice restored the intestinal recruitment of neutrophils after mucositis induction, stimulation of GF mice with a bacterial component (LPS) that generally stimulates the Myd88 signal did not induce neutrophil recruitment into the intestine of GF mice after irinotecan treatment.

It is well known that slgA protects the intestinal barrier, restricting the access of micro-organisms and antigens to the mucosal surface. This immunoglobulin also modulates the sampling of antigens and improves the quality of immune responses (Pabst, 2012). The higher production of slgA in CV mice than in GF mice is also well known, since only the former are exposed to the high variety of antigens of its microbiota (Sommer & Bäckhed, 2013). The low production of slgA observed in GF animals explains, in part, the innate inability of these animals to deal with various infectious agents or even other antigens (Hapfelmeier et al., 2010). During chemotherapies, the intestinal indigenous bacteria can be involved in sepsis episodes in which these bacteria take advantage of the increased intestinal permeability to translocate (Brandi et al., 2006). Inversely, studies have found that commensal microbiota produce metabolites that could be directly involved in a reduction of irinotecan toxicity (Lin et al.,...
2014). These findings show the dual role of microbiota on mucositis and reinforce the need for more studies to clarify these effects.

During cancer therapy with irinotecan, intestinal epithelial cells undergo considerable stress due to exposure to its cytotoxic metabolite SN-38, which acts as an inhibitor of topoisomerase I, leading to inhibition of both DNA replication and transcription (Voigt et al., 1998). After its action, SN-38 is inactivated by glucuronidation by the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) and eliminated in the bile as its glucuronidated form SN-38G (Sparreboom et al., 1998). However, this inactivated form might be re-activated in the intestine if bacteria producing β-glucuronidase are present in the local microbiota (Takasuna et al., 1996), although, in CV animals, most of the SN-38 produced from SN-38G by action of β-glucuronidase is rapidly adsorbed on the bacteria cell wall or intestinal dietary fibre, so that only 10 % remains in the unbound form that can be measured in the intestinal fluid (Takakura et al., 2012). This could explain, in part, the paradoxical higher levels of SN-38 observed in the intestinal luminal fluid of GF mice. Alternatively, it is well known that the indigenous microbiota stimulates the epithelial intestinal renewal, increasing cell sensitivity and/or the affinity of the intestinal cells to the drug and increasing its absorption. Indeed, the intestinal epithelial renewal rate is lower in GF animals when compared to CV mice (McLaughlin et al., 1964).

In this way, we hypothesized that the influence of the intestinal microbiota in the development of mucositis is also attributed to an unbalanced population (dysbiosis). Intestinal dysbiosis seems to be an important factor related to the pathogenesis of mucositis. Recently, it has been shown that chemotherapy treatment is associated with a deregulated intestinal microbial homeostasis and a decreased microbial diversity (van Vliet et al., 2009). Interestingly, an increasing amount of β-glucuronidase bacteria was found in mice with mucositis, and this increase can be considered as an indicator of dysbiosis (Stringer et al., 2007, 2009b). Consistent with these previous results, our data show that monoassociation of GF mice with E. coli producing β-glucuronidase (TGI) seems to have a direct relationship with the increase of intestinal permeability, but does not alter other parameters, such as recruitment of neutrophils and eosinophils, nor histology. In this sense, some authors suggest that other mechanisms besides the enzyme activity could be involved, such as Kurita et al. (2011) who carried out a study in Gunn rats treated with CPT-11. These animals have an inherent deficiency in the UGT1A1 enzyme and they do not perform the conjugation of SN-38 to SN-38G. In their study, the authors showed that mucositis was alleviated by antibiotic treatment, suggesting a bacterial involvement, but not specifically linked to β-glucuronidase. Furthermore, in a preliminary study, Fittkau et al. (2004) did not confirm the anti-diarrhoeal activity of D-saccharic acid 1.4-lactone (a specific β-glucuronidase inhibitor), although Wallace et al. (2010) showed an opposite result by oral administration of an inhibitor of the enzyme, which protected mice from irinotecan-induced toxicity. Takasuna et al. (1996) also showed that inhibition of the β-glucuronidase activity of the intestinal microbiota could be the major protective mechanism of antibiotics. Thus, the relationship between β-glucuronidase activity and mucositis is still controversial. Nevertheless, we demonstrate here that β-glucuronidase has only a partial relationship with some physiological disorders in the small intestine, indicating that other mechanisms are involved in the development of irinotecan-induced mucositis.

Thus, we conclude that the intestinal microbiota plays a significant role in the development and severity of irinotecan treatment. However, mechanisms other than β-glucuronidase are involved in the process, and further research is needed in order to propose alternative therapies, such as gut microbiota modulation, to increase the quality of life of patients with mucositis.

ACKNOWLEDGEMENTS

The authors thanks Dr Jamila Anba-Mondoloni, Unité Mixte de Recherche MICALIS, Equipe FLEC (Flore Lactique et Ecosystème Viande), Centre de Recherche INRA Jouy-en-Josas, France, for kindly providing the E. coli strains used in this work. The authors are grateful to Clelia N. Silva for valuable technical help, and to Marcelo Gomes and Valner Mussel for the animal care. This work was supported by grants from the Brazilian National Council for Scientific and Technological Development (CNPq), Ministry of Science and Technology (MCT), and the Foundation for Research Support of the State of Minas Gerais (FAPEMIG), Brazil, (APQ-00593-14). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES


Role of gut microbiota in irinotecan treatment


Edited by: A. van Vliet